

## **T Cell Priming Versus T Cell Tolerance Induced by Synthetic Peptides**

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### **Summary**

It is well known that synthetic peptides are able to both induce and tolerize T cells. We have examined the parameters leading either to priming or tolerance of CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) in vivo with a major histocompatibility complex class I (H-2 D<sup>b</sup>) binding peptide derived from the glycoprotein (GP aa33-41) of lymphocytic choriomeningitis virus (LCMV). By varying dose, route, and frequency of LCMV GP peptide application, we found that a single local subcutaneous injection of 50–500 µg peptide emulsified in incomplete Freund's adjuvant protected mice against LCMV infection, whereas repetitive and systemic intraperitoneal application of the same dose caused tolerance of LCMV-specific CTL. The peptide-induced tolerance was transient in euthymic mice but permanent in thymectomized mice. These findings are relevant for a selective use of peptides as a therapeutic approach: peptide-induced priming of T cells for vaccination and peptide-mediated T cell tolerance for intervention in immunopathologies and autoimmune diseases.

Proteins may serve as immunogens or tolerogens, depending on mode of administration. Several factors determine whether a protein induces a T cell response or renders a T cell unresponsive: amount of antigen, route and frequency of application, use of adjuvants, and organization of the antigen (1–3). Since T cells recognize endogenously processed peptides presented by MHC class I or class II molecules (4–7), the question arises whether the rules for priming and tolerance induction with protein antigens also apply for synthetic peptides. Several reports have successfully demonstrated T cell immunity induced with peptides in vivo (8–14). These findings, together with the development of methods to identify naturally processed peptides and to predict peptide binding motifs for MHC molecules (15–18), opened up the possibility of using synthetic peptides as safe vaccines (19). Recent reports however, have also documented that peptide antigens may be used to downregulate T cell-mediated immune responses (20–23). Thus, peptide-induced T cell tolerance may represent a tool to prevent or even treat T cell-mediated autoimmune diseases (24–32). If peptides are able to induce both immunity and tolerance, how can T cell priming be achieved without inducing tolerance, and how can tolerance be established without activation of T cells? We have analyzed this issue by using a synthetic peptide derived from the glycoprotein (GP aa33-41 = GP33 peptide) of lymphocytic choriomeningitis virus (LCMV) and by varying peptide dose (1–1,000 µg), route (i.p. versus s.c.), and frequency (single versus repetitive) of application. The aim of the study was to define

protocols for peptide applications which either result in priming or tolerance of peripheral CD8<sup>+</sup> CTL.

### **Materials and Methods**

**Animals.** C57BL/6 (H-2<sup>b</sup>) mice were purchased from the Institut für Zuchtthgiene (Tierspital Zürich, Switzerland). H-2<sup>b/d</sup> mice were obtained by breeding C57BL/6 (H-2<sup>b</sup>) mice with B10.D2 (H-2<sup>d</sup>) mice. Animals were kept under conventional conditions and used in the experiments 10–14 wk after birth. C57BL/6 mice were thymectomized at 5–10 wk of age as described (33) and used 4–8 wk after thymectomy.

**Virus.** The LCMV-WE strain was originally obtained from Dr. F. Lehmann-Grube (Hamburg, Germany) and was propagated in our laboratory on L929 fibroblast cells (34). Virus stocks were diluted in MEM supplemented with 2% FCS. Mice were infected i.v. with 200 PFU of LCMV-WE.

**Peptide Treatment.** LCMV glycoprotein peptide GP33-41 (KAVYNFATM) (35) and LCMV nucleoprotein peptides NP 394-408 (AIFQPQNGQFIHFYR) and NP118-126 (RPQASGVYM) (36) were synthesized by a solid-phase method and purchased from Neosystem Laboratoire (Strasbourg, France). For technical reasons (to prevent dimer formation), the original cysteine at the anchor position 41 in the LCMV glycoprotein peptide was replaced by methionine. For injections, peptides were dissolved in HBSS and given either i.v. or i.p. in a total volume of 200 µl or emulsified 1:1 (vol/vol) in IFA (Difco Laboratories, Detroit, MI) and injected either s.c. at the base of the tail or i.p. in a total volume of 200 µl. Repetitive peptide injections were done at 3-d intervals.

**Virus Protection Assay.** Mice were treated with different peptide immunization protocols as indicated. 10 d after the last peptide administration, mice were challenged i.v. with 200 PFU LCMV-WE and the virus titer in the spleen was determined 4 d later as described in detail (37).

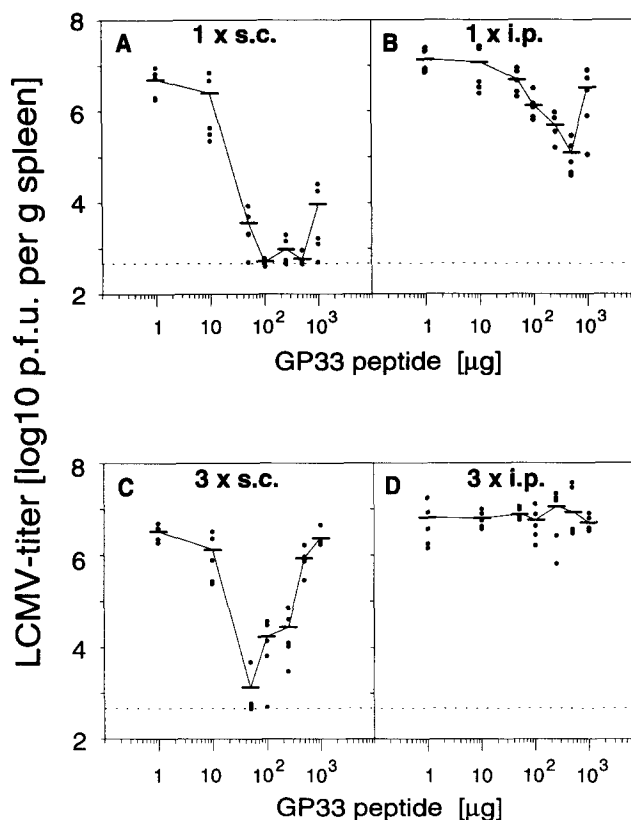
**Cytotoxicity Assay.** Single spleen cell suspensions from mice infected with LCMV-WE 8 d before testing were prepared in complete MEM. The cytolytic activity was determined in a  $^{51}\text{Cr}$  release assay as described (38). Briefly, fibroblast target cells MC57G (H-2<sup>b</sup>) and D2 (H-2<sup>d</sup>) were coated with LCMV peptides GP33-41, NP394-408, or NP118-126 at a concentration of  $10^{-6}$  M, and were labeled with 250  $\mu\text{Ci}$   $^{51}\text{Cr}$  for 2 h at 37°C on a rocking platform. Target cells were washed three times and counted.  $10^4$  target cells were incubated in 96-well round-bottom plates with spleen effector cells at ratios of 70:1, 23:1, 8:1, and 3:1 in a final volume of 200  $\mu\text{l}$ . After a 5-h incubation period at 37°C, 70- $\mu\text{l}$  supernatants were harvested and assayed. Spontaneous release was always <20%.

## Results

**Peptide-induced Priming of LCMV-specific CTL.** The priming capacity of the LCMV GP33 peptide was examined in a virus protection assay: C57BL/6 mice (H-2<sup>b</sup>) were immunized once or repetitively with different doses of GP33 peptide in IFA by different routes (s.c. versus i.p.). 10 d after the last peptide application, mice were challenged with LCMV, and 4 d later, LCMV titers in the spleen were determined. Infection of naive C57BL/6 mice with 200 PFU LCMV caused a high virus titer ( $>10^7$  PFU/g spleen), whereas mice previously ( $>30$  d) infected with LCMV were able to rapidly clear virus upon a second LCMV infection (virus titer below detection level: <500 PFU/g spleen). Mice injected once s.c. with 50–500  $\mu\text{g}$  of GP33 peptide emulsified in IFA exhibited low virus titer ( $10^3$  PFU/g) comparable to LCMV-primed memory mice (Fig. 1 A). In contrast, a single i.p. injection of GP33 peptide in IFA caused only moderate protection ( $10^5$  PFU/g) despite the high dose of peptide (250–500  $\mu\text{g}$ ) injected (Fig. 1 B).

In a second experiment, the GP33 peptide was given to C57BL/6 mice three times at 3-d intervals either s.c. or i.p. (Fig. 1, C and D). Subcutaneous application of  $3 \times 50$   $\mu\text{g}$  GP33 peptide yielded complete protection ( $10^3$  PFU/g). With increasing peptide doses, however, protection vanished (Fig. 1 C). In contrast, mice treated three times i.p. with GP33 peptide were not protected against LCMV at any dose of peptide tested (Fig. 1 D).

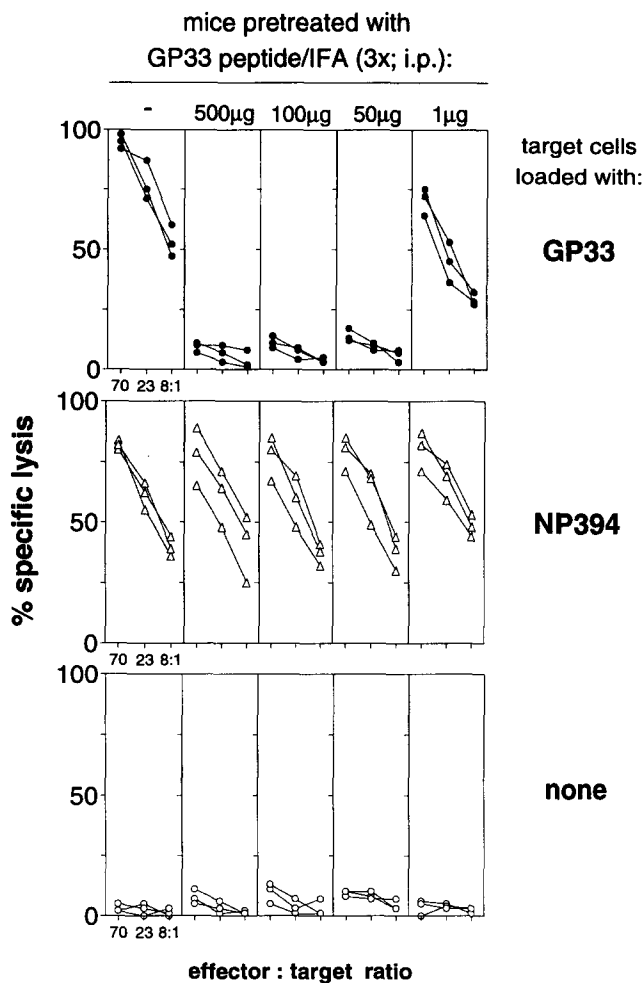
**Peptide-induced Tolerance of CTL.** Failure to induce protection with repetitive i.p. administration of GP33 peptide suggested that peptide treatment caused tolerance of GP33-specific CTL. To test this, the LCMV-specific CTL activities of peptide-treated mice were determined 8 d after LCMV infection. LCMV-infected control C57BL/6 mice, pretreated with IFA only, were able to generate CTL specific for target cells loaded either with the GP33 or NP394 peptide (Fig. 2, far left panels). The LCMV nucleoprotein peptide NP394-408 (NP394 peptide) is an additional CTL epitope in H-2<sup>b</sup> mice that allowed us to control the specificity of GP33 peptide-induced tolerance. As shown in Fig. 2, mice treated three times i.p. with 50–500  $\mu\text{g}$  of GP33 peptide in IFA failed to



**Figure 1.** Parameters defining peptide-induced priming or tolerance of CTL. C57BL/6 mice were immunized with different amounts of GP33 peptide emulsified in IFA once s.c. (A) or i.p. (B) or three times at 3-d intervals s.c. (C) or i.p. (D). 10 d after the last peptide administration, mice were challenged i.v. with 200 PFU LCMV-WE, and virus titer in the spleen was analyzed 4 d later. Dots represent values from individual mice (five animals per group). Detection level is indicated as a dotted line.

mount a GP33-specific CTL response after LCMV infection. In contrast, the NP394-specific CTL response was not affected by the GP33 peptide treatment. Thus, tolerance induction in vivo was specific and not caused by an unspecific toxic or general immunosuppressive effect of the GP33 peptide application. As indicated in Fig. 2, the minimal amount of GP33 peptide required for tolerance induction by i.p. administration ranged between 1 and 50  $\mu\text{g}$ .

To evaluate the time dependence of peptide-induced tolerance, the LCMV GP33-specific CTL response was determined in peptide-pretreated mice ( $3 \times 200$   $\mu\text{g}$  GP33 peptide/IFA; i.p.) at different time points after the treatment with peptide. As shown in Fig. 3 A, the GP33-specific CTL response was virtually absent in peptide-treated mice  $\leq 3$  wk after tolerization. Afterwards, however, tolerance vanished and 7 wk after peptide treatment, the GP33-specific CTL activities were comparable to controls. Thus, peptide-induced CTL tolerance was complete but transient in normal euthymic mice. In contrast, thymectomized mice exhibited no GP33-specific CTL response, even 30 wk after peptide treatment (Fig. 3 B). This finding indicates that the loss of tolerance in euthymic mice was due to the generation of “new” GP33-specific CTL in the thymus when the in vivo concentration of GP33

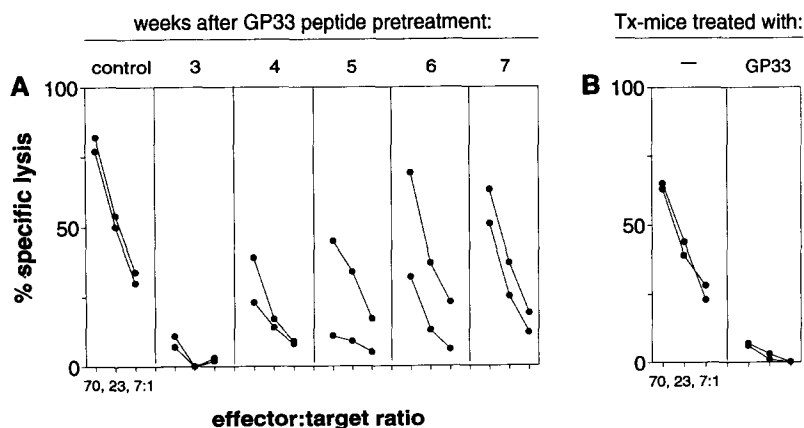


**Figure 2.** Peptide-induced tolerance of CTL. C57BL/6 mice were treated three times at 3-d intervals with 1–500  $\mu\text{g}$  GP33 peptide in IFA i.p. or with IFA alone. Lines represent individual animals. 10 d after the last peptide application, mice were infected i.v. with 200 PFU LCMV-WE, and the ex vivo CTL activities of splenocytes were assayed 8 d later in a  $^{51}\text{Cr}$  release assay. MC57G fibroblast target cells were either loaded with  $10^{-6}$  M GP33 peptide ( $\bullet$ ) or with  $10^{-6}$  M NP394 peptide ( $\Delta$ ) or left untreated ( $\circ$ ).

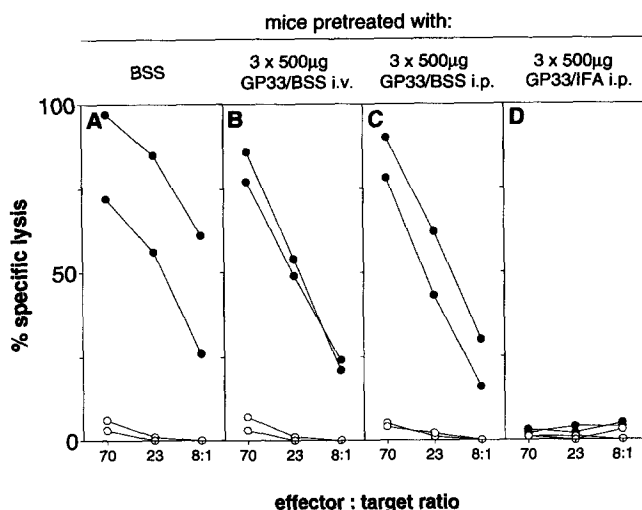
peptide had dropped below a certain threshold. It further reveals that neither induction nor maintenance of the GP33 peptide-induced CTL tolerance depends upon the thymus.

**Role of Adjuvants in Induction of CTL Tolerance.** To define the role of adjuvants (IFA) in tolerance induction, a tolerogenic dose of GP33 peptide (500  $\mu\text{g}$ ) was administered three times at 3-d intervals i.v. or i.p. without IFA (Fig. 4, B and C). In both cases, the GP33-specific CTL response was not reduced 8 d after LCMV infection, when compared to BSS-treated control animals (Fig. 4 A). In contrast, mice treated three times with 500  $\mu\text{g}$  GP33 peptide emulsified in IFA exhibited no GP33-specific CTL activity (Fig. 4 D). Tolerance induction without adjuvants was only achieved when high amounts (>500  $\mu\text{g}$ ) of GP33 peptide were coadministered i.v. and i.p. (data not shown). The adjuvants most likely caused a slow release and prevented rapid degradation of the peptide, thereby prolonging its half-life in vivo.

**Specific CTL Tolerance Induced by NP118 Peptide or GP33 Peptide.** To demonstrate that peptide-induced CTL tolerance was not a special feature of GP33 peptide, the LCMV-specific CTL response of (C57BL/6  $\times$  B10.D2) $F_1$  mice was examined. These mice are able to develop an H-2<sup>b</sup>-restricted GP33-specific and an H-2<sup>d</sup>-restricted NP118-specific CTL response after LCMV infection. H-2<sup>b/d</sup>  $F_1$  mice were treated i.p. three times at 3-d intervals with GP33 peptide (500  $\mu\text{g}$ ) or with NP118 peptide (500  $\mu\text{g}$ ). IFA-treated control animals showed high CTL activities against both H-2<sup>b</sup> target cells loaded with GP33 peptide and H-2<sup>d</sup> target cells loaded with NP118 peptide (Fig. 5 A). Mice injected with GP33 peptide did not develop a GP33-specific CTL response, whereas the NP118-specific CTL response was within normal ranges (Fig. 5 B). Correspondingly, the NP118-specific CTL activity in NP118 peptide-treated mice was strongly reduced, whereas the GP33-specific CTL activity was not diminished (Fig. 5 C). Thus, the ability of a MHC class I binding peptide to tolerate T cells is demonstrated for two independent cases.



**Figure 3.** Peptide-induced T cell tolerance is transient in euthymic mice. (A) C57BL/6 mice, injected i.p. three times at 3-d intervals with 200  $\mu\text{g}$  GP33 peptide/IFA or IFA alone, were infected i.v. with 200 PFU LCMV-WE at different time points after the last peptide application. Primary CTL activities of splenocytes were determined at the indicated weeks after tolerance induction on MC57G target cells loaded with  $10^{-6}$  M GP33 peptide ( $\bullet$ ). (B) Thymectomized (Tx) mice were treated the same way and were challenged with LCMV 30 wk later.



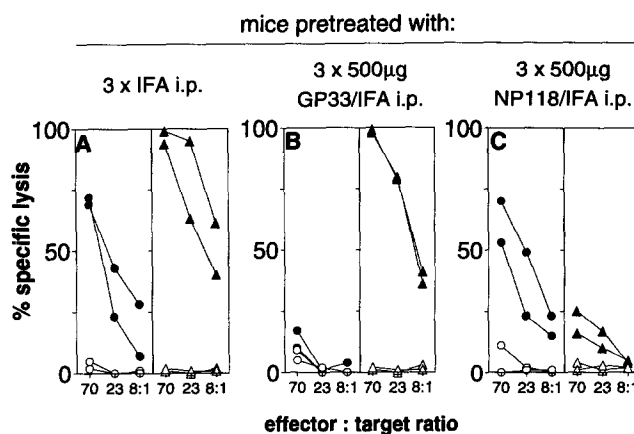
**Figure 4.** Role of adjuvants in inducing tolerance. C57BL/6 mice were injected three times at 3-d intervals with 200  $\mu$ l BSS (A) or with 500  $\mu$ g of free GP33 peptide dissolved in BSS i.v. (B) or i.p. (C). Another group of mice was treated i.p. with 500  $\mu$ g of GP33 peptide emulsified in IFA (D). 10 d after the last peptide injection, mice were challenged i.v. with 200 PFU LCMV-WE and primary CTL activities of splenocytes were determined 8 d later. MC57G target cells were left untreated (○) or loaded with  $10^{-6}$  M GP33 peptide (●).

## Discussion

In the present report, we have defined protocols for peptide-mediated T cell priming or tolerance: 50–500  $\mu$ g of GP33 peptide injected once s.c. generated a protective CTL response without inducing tolerance (Fig. 1 A), whereas the same dose of the same peptide administered three times i.p. tolerized the specific CTL without evidence of an immunizing effect (Fig. 1 D). How can these opposing effects of peptide injection on T cells be explained?

Subcutaneous application of peptide in IFA generates an antigen depot from where the peptide slowly reaches the draining lymph nodes. There peptide-loaded professional APCs are able to induce optimal T cell activation by providing both TCR engagement and costimulatory signal (39, 40). The slow release of peptide from a local depot (41), probably together with inflammatory reactions in the skin caused by the injection procedure, may represent additional factors for optimal T cell priming. Once sufficiently activated, increased numbers of peptide-specific T cells may leave the regional lymph nodes and circulate through the body.

In marked contrast, when administered intraperitoneally or when high amounts ( $3 \times 500$ – $1000$   $\mu$ g) of GP33 peptide



**Figure 5.** Specific CTL tolerance induced by NP118 peptide or GP33 peptide. (C57BL/6  $\times$  B10.D2) $F_1$  mice were treated i.p. three times at 3-d intervals with either IFA (A) or 500  $\mu$ g GP33 peptide in IFA (B) or 500  $\mu$ g NP118 peptide in IFA (C). 10 d after the last peptide application, mice were infected i.v. with 200 PFU LCMV-WE and CTL activities of splenocytes were tested on GP33-loaded (●) or unloaded (○) MC57G (H-2<sup>b</sup>) or on NP118-loaded (▲) or unloaded (△) D2 (H-2<sup>d</sup>) target cells.

were injected s.c., the peptide is distributed throughout the whole body. Thus, most if not all peptide-specific T cells are triggered via their TCR by neighboring cells that are not exclusively professional APCs and therefore may not provoke costimulation (42). When given the peptide systemically, it is likely that activated T cells reencounter antigen in form of peptide-coated cells within a short time after initial triggering. Since T cells that are in cell cycle undergo apoptosis after TCR-mediated stimulation (43), one could imagine that most peptide-activated T cells may succumb to activation-induced cell death when the peptide antigen is present ubiquitously. Alternatively, peptides given systemically and repetitively may induce virtually all specific T cells to become terminally differentiated effector T cells that will die within a few days (44, 45). Thus, lack of costimulation, activation-induced cell death, and exhaustion may contribute to peptide-induced peripheral expansion and deletion of peptide-specific T cells, as observed in TCR transgenic mice (22, 46–48).

Our findings hold implications for the design of peptide therapies to induce CTL for vaccination against tumors (49–51) and infectious agents (9, 52–54) or to downregulate CTL activities in T cell-dependent diseases (27, 32). The study provides the experimental framework to separate the immunogenic and tolerogenic effect of peptide administration on CD8<sup>+</sup> CTL in vivo.

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